

# Clonal deletion of thymocytes can occur in the cortex with no involvement of the medulla

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The thymic medulla is generally held to be a specialized environment for negative selection. However, many self-reactive thymocytes first encounter ubiquitous self-antigens in the cortex. Cortical epithelial cells are vital for positive selection, but whether such cells can also promote negative selection is controversial. We used the HY<sup>cd4</sup> model, where T cell receptor for antigen (TCR) expression is appropriately timed and a ubiquitous self-antigen drives clonal deletion in male mice. We demonstrated unambiguously that this deletion event occurs in the thymic cortex. However, the kinetics *in vivo* indicated that apoptosis was activated asynchronously relative to TCR activation. We found that radioresistant antigen-presenting cells and, specifically, cortical epithelial cells do not efficiently induce apoptosis, although they do cause TCR activation. Rather, thymocytes undergoing clonal deletion were preferentially associated with rare CD11c<sup>+</sup> cortical dendritic cells, and elimination of such cells impaired deletion.

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Abbreviations used: cTEC, cortical thymic epithelial cell; DN, double negative; DP, double positive; DTR, diphtheria toxin receptor; DTx, diphtheria toxin; mTEC, medullary thymic epithelial cell.

Although T lymphocytes play a central role in the adaptive immune response to foreign antigens, they are also capable of attacking and destroying normal tissue and driving autoimmunity. Negative selection exists as a mechanism of central tolerance to deal with autoreactive T cell clones (for review see reference 1). It is primarily achieved by clonal deletion or the induction of apoptosis in thymocytes with a high affinity for self-peptide/MHC. Despite the importance of clonal deletion, many fundamental questions remain, such as the following: at which stages of development do thymocytes undergo deletion; where does this correspond to anatomically; and what cell types induce apoptosis?

The use of different model systems in previous studies aimed at answering these questions has led to conflicting results. The earliest experiments studying negative selection used endogenous superantigens to induce a high-affinity signal. However, this system may provide a qualitatively different signal than TCR ligation by self-peptide/MHC because of the nature of superantigen recognition. Injection of TCR

cross-linking antibodies to simulate a high-affinity signal has also been used (for review see reference 1). However, this model is unlikely to mimic antigen-specific deletion, as it results in high-level glucocorticoid production that causes nonspecific thymocyte death (2). The development of TCR transgenics was a major improvement and allowed for the study of negative selection in response to either exogenous peptide injection or endogenous high-affinity self-antigens (3). Importantly though, WT thymocytes do not express a surface TCR until the double-positive (DP) stage when they rearrange their TCR $\alpha$  loci, whereas conventional TCR transgenics express both the TCR $\alpha$  and TCR $\beta$  chains at the early double-negative (DN) stage. Consequently, negative selection occurs prematurely in TCR transgenics (4), and when the anatomical location of clonal deletion was specifically examined using the F5 TCR transgenic, apoptotic thymocytes were found to be located at the cortico-medullary junction (5). More recent studies have made it clear that early

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expression of a TCR transgene (6–8) and the high frequency of antigen-specific T cells in TCR transgenics (9, 10) cause a variety of nonphysiological effects. To highlight this point, the HY TCR transgenic model, which has been used extensively to study negative selection, develops an aberrant population of thymocytes that are prematurely selected because of early TCR expression (11).

The medulla is considered a specialized site for negative selection because of AIRE-mediated expression of tissue-restricted antigens (12), as well as its abundance of DCs that express a high level of costimulatory molecules such as B7-1/2 and CD40 (13–16). Indeed, several strains of mice that lack an organized medulla have severe autoimmune disease (17–20). Furthermore, mice deficient for CCR7, a chemokine receptor which is important for migration to the medulla (21), develop autoimmune pathology, a result which may be caused by a combination of defective regulatory T cell generation (22), insufficient TCR tuning (23), or incomplete clonal deletion of autoreactive thymocytes (24). Despite the compendium of work implicating the medulla as the primary site of clonal deletion, many thymocytes are predicted to be reactive to ubiquitous self-antigens and would first encounter them in the cortex.

Cortical thymic epithelial cells (cTECs) are the predominant stromal cell in the cortex and are essential for positive selection (for review see reference 25). Some studies have suggested that cTECs fail to induce tolerance to self-antigens (26–30), whereas others have come to the opposite conclusion (31–40). Thus, the role of cTECs in central tolerance is poorly understood.

Given this information, we decided to revisit the process of clonal deletion by using the HY<sup>cd4</sup> model, where antigen-induced negative selection occurs at the DP stage (41). For the first time, we were able to visualize DP thymocytes undergoing clonal deletion in response to an endogenous and ubiquitous self-antigen *in vivo*. Surprisingly, thymocytes undergoing clonal deletion were found throughout the cortex and were absent from the medulla altogether. Male-reactive DP thymocytes could be recovered in relatively high numbers from these mice because of an asynchronous induction of apoptosis. This was likely because of the fact that cTECs, although able to trigger TCR activation, did not efficiently induce apoptosis. As a possible explanation for the delayed induction of apoptosis, we show that apoptotic cells were preferentially associated with CD11c<sup>+</sup> DCs present throughout the cortex. These results suggest a model where DP thymocytes with high affinity for a ubiquitous self-antigen are first signaled in the cortex but require further stimuli from DCs to undergo clonal deletion efficiently.

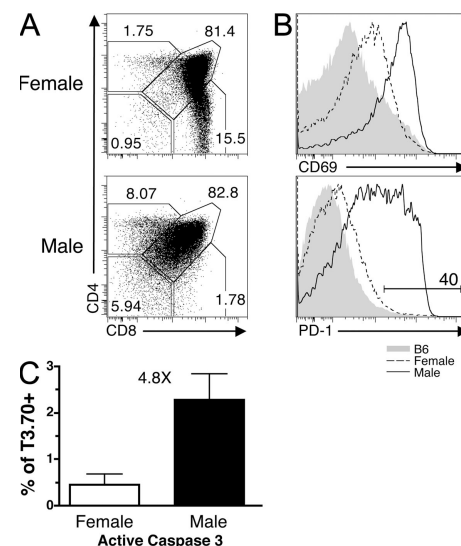
## RESULTS

### The phenotype and kinetics of HY<sup>cd4</sup> thymocytes undergoing clonal deletion

Developing thymocytes are not competent to undergo clonal deletion until they express a TCR $\alpha\beta$  heterodimer, an event which occurs primarily at the DP stage of development. Con-

ventional TCR transgenics do not recapitulate the appropriate timing of TCR $\alpha$  expression. Therefore, we made use of the HY<sup>cd4</sup> model, where expression of the TCR $\alpha$  chain is delayed until the DN to DP transition. This allowed us to examine the consequence of high-affinity TCR ligation on DP thymocytes. Similar to the conventional HY TCR transgenic, HY<sup>cd4</sup> thymocytes are T3.70<sup>+</sup> and are positively selected in female mice but have high affinity for a male antigen and undergo clonal deletion in male mice. Importantly, deletion in male mice is strikingly different from the conventional HY model because deletion occurs at the DP stage (41). In HY<sup>cd4</sup> male mice, DP thymocytes failed to form a CD8 SP population and instead dramatically dulled their CD4 and CD8 expression (Fig. 1 A) and up-regulated CD69 and PD-1 to levels higher than in the female (Fig. 1 B). To monitor the activation of the apoptosis pathway, as a measure of clonal deletion, we used an antibody specific for the active form of Caspase 3 (42). Despite the fact that macrophages engulf apoptotic cells in the thymus (43), active Caspase 3<sup>+</sup> cells were detected in HY<sup>cd4</sup> male mice at a 4.8-fold greater frequency than in female mice in the steady state (Fig. 1 C). Activation of Caspase 3 appears to be a relevant measure of clonal deletion because in male HY<sup>cd4</sup> mice on a Bim<sup>o</sup> background, we saw that Caspase 3 was not activated to a level higher than that of HY<sup>cd4</sup> females (Fig. S1, available at <http://www.jem.org/cgi/content/full/jem.20080866/DC1>).

Despite the fact that male-reactive cells undergo clonal deletion in male mice, a substantial population of antigen-experienced (CD69<sup>+</sup>) T3.70<sup>+</sup> DP cells was detectable in male mice.



**Figure 1. The phenotype of thymocytes undergoing positive and negative selection in the HY<sup>cd4</sup> model.** (A) CD4 by CD8 profiles of T3.70<sup>+</sup> thymocytes from female and male mice. (B) The expression of CD69 and PD-1 on thymocytes of the indicated genotype. The percentage of PD-1<sup>+</sup> cells in the HY<sup>cd4</sup> male is indicated. (C) The activation of Caspase 3 in male mice compared with female mice. The fold change in male over female is indicated. Data represent the mean from 16 individuals from 12 different experiments  $\pm$  SD.  $P < 0.0001$ .

To explain this observation, we hypothesized that apoptosis must occur sufficiently long enough after TCR engagement to allow signaled thymocytes to accumulate in the steady state. To address this question, we examined the kinetics of clonal deletion *in vivo* by performing a BrdU time course. BrdU labels dividing thymocytes at the DN to DP transition, which corresponds to the stage when HY<sup>cd4</sup> thymocytes begin to express their heterodimeric TCR. We used a “pulse-chase” strategy where BrdU labeled a cohort of cells, which were then followed over time. This strategy is particularly effective in the thymus because the label is rapidly incorporated, not reutilized, and free label is not available very long (44).

2 h after BrdU injection, the majority of labeled thymocytes in both female and male HY<sup>cd4</sup> mice were DP (Fig. 2 A), as in WT mice (44). Approximately half of these were T3.70<sup>+</sup> in both female and male mice (unpublished data). Although the T3.70<sup>+</sup> BrdU<sup>+</sup> DPs in female mice did not express CD69 at the 2-h time point, we were surprised to observe that ~60% of the T3.70<sup>+</sup> BrdU<sup>+</sup> DPs were already CD69<sup>+</sup> in male mice

(Fig. 2 B). This indicates a very rapid and efficient response to male antigen very early in the lifespan of the DP thymocyte. Indeed, by 12 h, most labeled T3.70<sup>+</sup> DPs expressed a high level of CD69 in male mice. Because there was not a substantial lag in either the expression of HY TCR $\alpha$  or response to male antigen in labeled cells, this was an effective means to measure the kinetics of clonal deletion *in vivo*. Importantly, we also noted the presence of T3.70<sup>+</sup> BrdU<sup>+</sup> DPs that were CD69<sup>-</sup> in male mice at the earliest time points, which suggests that the cd4-mediated expression of the TCR $\alpha$  transgene is in fact delayed until the transition of thymocytes to the DP stage.

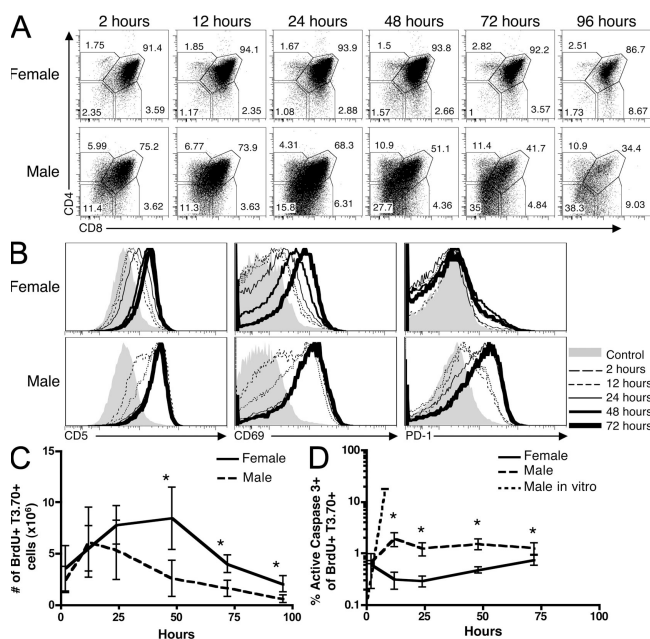
The total number of BrdU<sup>+</sup> T3.70<sup>+</sup> DPs in the female and male mice reached a maximum after ~24 h. In female mice, these cells remained for 48 h and began to decrease by 72 h (Fig. 2 C) because of positive selection and differentiation to CD8 SP (Fig. 2 A). In contrast, the numbers of BrdU<sup>+</sup> T3.70<sup>+</sup> cells began to decrease in the male mice (relative to the female) after 24 h and continued over the next 3 d (Fig. 2 C). This was accompanied by further dulling of CD4 and CD8, with an increase in the percentage of cells falling in the DN gate and a concomitant decrease in cells in the DP gate (Fig. 2 A).

CD5 and CD69 were both up-regulated more quickly in the male than in the female and also to a higher level. PD-1 is an inhibitory receptor that is up-regulated in the male after a high-affinity signal but is not up-regulated in the female (45). The up-regulation of PD-1 was slower than that of CD5 and CD69 in the male, reaching a maximum only after 48 h (Fig. 2 B). In the steady state, ~40% of T3.70<sup>+</sup> thymocytes are PD-1<sup>+</sup> in male mice (Fig. 1 B). We interpret this to mean that at least some of T3.70<sup>+</sup> thymocytes in HY<sup>cd4</sup> males are up to 48 h old in the steady state.

We also examined the activation of Caspase 3 to assess clonal deletion. A small but significant fraction of BrdU<sup>+</sup> T3.70<sup>+</sup> cells activated Caspase 3 in the male compared with female (1.5 vs. 0.3%; Fig. 2 D). This difference was first apparent at 12 h but remained for the duration of the experiment. This indicates that apoptosis does not occur in a synchronous fashion after antigen encounter *in vivo*. The fact that a fraction of both PD-1<sup>-</sup> and PD-1<sup>+</sup> cells expressed active Caspase 3 (unpublished data) also indicates asynchronous induction of apoptosis. In contrast, when we used a similar approach to quantify apoptosis of HY<sup>cd4</sup> thymocytes exposed to male antigen *in vitro*, we did observe synchronous deletion, with 17% of T3.70<sup>+</sup> cells being active Caspase 3<sup>+</sup> by 8 h (Fig. 2 D). Altogether, these data illustrate that *in vivo* clonal deletion occurs asynchronously and inefficiently and allows for the accumulation of signaled male-reactive thymocytes in the steady state.

### Mixed BM chimeras as a strategy to study clonal deletion

To further examine the anatomical location of clonal deletion and factors involved, we sought a system that allowed us to easily test various genetic deficiencies and to limit antigen presentation to particular subsets of cells. To do this, we used a mixed BM chimera strategy where HY<sup>cd4</sup> female BM was mixed at a low ratio with competitor BM from WT mice and used to reconstitute female or male recipients. This experimental

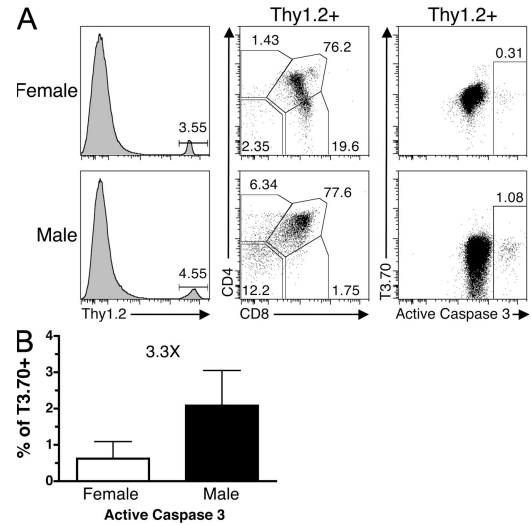


**Figure 2. Apoptosis occurs asynchronously *in vivo*, with some cells surviving up to 4 d.** BrdU was injected i.p. at the indicated number of hours before harvest. (A) The expression of CD4 and CD8 on BrdU<sup>+</sup> T3.70<sup>+</sup> thymocytes from representative mice from the indicated time point. (B) Expression of CD5, CD69, and PD-1 on BrdU<sup>+</sup> T3.70<sup>+</sup> thymocytes over time. (C) The total number of BrdU<sup>+</sup> T3.70<sup>+</sup> thymocytes versus time after BrdU injection. Data represent the mean  $\pm$  SD from four separate time courses including three to seven individual mice. \*,  $P = 0.0118$  at 48 h,  $P = 0.0070$  at 72 h, and  $P = 0.0037$  at 96 h. (D) The frequency of active Caspase 3<sup>+</sup> cells as a percentage of BrdU<sup>+</sup> T3.70<sup>+</sup> thymocytes. Data represent the mean  $\pm$  SD from four separate time courses including three to seven individual mice. \*,  $P = 0.0002$  at 12 h,  $P < 0.0001$  at 24 h,  $P = 0.0004$  at 48 h, and  $P = 0.0061$  at 72 h. Activation of Caspase 3 *in vitro* was determined by stimulating female HY<sup>cd4</sup> thymocytes with HYp peptide plus spleen APC for 8 h. Data were normalized for nonspecific death caused by *in vitro* culture and represent the mean  $\pm$  SD from triplicate wells.

strategy has the additional advantage that the precursor frequency is reduced, thereby reducing clonal competition and correcting thymic architecture defects, which are typical of TCR transgenics (46, 47). In addition, for some experiments we bred  $HY^{cd4}$  mice onto the nonselecting MHC Class I  $D^{bo}$ -deficient ( $D^{bo}$ ) background to eliminate the possibility of antigen presentation by  $HY^{cd4}$  donor-derived cells (Fig. S2, available at <http://www.jem.org/cgi/content/full/jem.20080866/DC1>). For a summary of mixed chimeras used, see Table I. To validate the use of the mixed BM chimera approach, we first examined the phenotype of  $HY^{cd4}$  cells undergoing positive selection and clonal deletion in mixed BM chimeras. When  $HY^{cd4}$  TCR $\alpha^0$  female BM was mixed at a low ratio with congenically marked B6.PL female or male BM and transferred into female or male B6.PL hosts, respectively, we saw characteristic positive and negative selection in female and male mice. This included typical CD4 by CD8 profiles and activation of Caspase 3 (compare Figs. 1 and 3).

#### $HY^{cd4}$ thymocytes undergo clonal deletion in the cortex with no involvement of the medulla

One hypothesis to explain why deletion is asynchronous in  $HY^{cd4}$  mice is that thymocytes must traffic to the medulla and receive further stimuli to undergo apoptosis. If this were the case, one would expect that  $HY^{cd4}$  cells in male mice would up-regulate CCR7 to facilitate migration to the medulla. However, we found that very few cells were CCR7<sup>+</sup> in male mice compared with female mice. Interestingly, when we bred  $HY^{cd4}$  mice onto a Bim-deficient background we saw that the frequency of CCR7<sup>+</sup> thymocytes increased in male mice (Fig. 4 A). These results suggested that CCR7<sup>+</sup> cells are preferentially undergoing apoptosis, and they would seem to support



**Figure 3.  $HY^{cd4}$  mixed BM chimeras as a model system to study clonal deletion in vivo.** (A)  $HY^{cd4}$  TCR $\alpha^0$  female BM was mixed with congenic B6.PL female and male BM and transferred into congenic female and male hosts. Chimeras were harvested at 6 wk and thymi were analyzed by flow cytometry for expression of Thy1.2, T3.70, CD4, CD8, and active Caspase 3.  $HY^{cd4}$  donor-derived cells were identified by Thy1.2 where indicated. Numbers represent frequency of parent gate. (B) The activation of Caspase 3 in male chimeras compared with female chimeras. The fold change in male over female is indicated. Data represent the mean from 15–17 individuals from six different experiments  $\pm$  SD.  $P < 0.0001$ .

the hypothesis that migration to the medulla is important for clonal deletion.

To directly test the role of CCR7 in clonal deletion, we bred the  $HY^{cd4}$  mice onto a CCR7-deficient background.

**Table I. A summary of mixed BM chimeras generated for these studies**

Mixed BM donors		Recipients	Male antigen-presenting cell type	Figure
(~5%)	(~95%)			
$HY^{cd4}$ TCR $\alpha^0$	B6.PL Female B6.PL Male	B6.PL Female B6.PL Male	None All	Figs. 3, 5, and 7
$HY^{cd4}$ or $HY^{cd4}$ $D^{bo}$	B6 Female B6 Male	B6 Female B6 Male	None All	Figs. 4, 6, S1, S3, S4, S5, and S6 (controls)
$HY^{cd4}$ Bim <sup>0</sup>	B6 Female B6 Male	B6 Female B6 Male	None All	Figs. 4 and S1
$HY^{cd4}$ CCR7 <sup>0</sup>	B6 Female B6 Male	B6 Female B6 Male	None All	Figs. 4 and S3
$HY^{cd4}$ $D^{bo}$	$D^{bo}$ Female	B6 Male	Radioresistant cells	Figs. 6 and S6
$HY^{cd4}$ $D^{bo}$	B6 Female	K14-HYp Female	cTECs	Figs. 6 and S6
$HY^{cd4}$ $D^{bo}$	$D^{bo}$	$D^{bo}$	None	Figs. 8 and S8
	CD11c-DTR Female	B6 Female	None	
	CD11c-DTR Male	B6 Female	BM-derived cells	
	CD11c-DTR Male	B6 Male	All	
$HY^{cd4}$ $D^{bo}$	<i>plt/plt</i> Female <i>plt/plt</i> Male	<i>plt/plt</i> Female <i>plt/plt</i> Male	None All	Fig. 4
$HY^{cd4}$ $D^{bo}$	<i>aly/aly</i> Female <i>aly/aly</i> Male	<i>aly/aly</i> Female <i>aly/aly</i> Male	None All	Fig. S5

A mixture of BM from the indicated mice was transferred into the indicated recipients to create chimeras where  $HY^{cd4}$  thymocytes were at a low ratio (~5%).

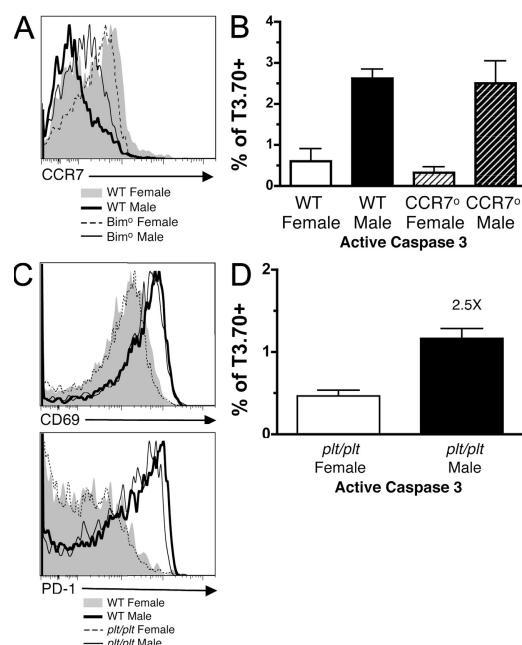


When these mice were used to create mixed BM chimeras, we saw no defect in clonal deletion. Specifically, no difference was seen between  $\text{HY}^{\text{cd}4}$  CCR7 $^{\circ}$  and WT thymocytes with respect to their CD4 by CD8 profiles, up-regulation of CD69 and PD-1 (Fig. S3, available at <http://www.jem.org/cgi/content/full/jem.20080866/DC1>), or activation of Caspase 3 (Fig. 4 B). As support for these findings, similar results were obtained with mixed chimeras with *plt/plt* mutant recipient mice (Fig. 4, C and D). *Plt/plt* mice are deficient for CCR7 ligands because of a deletion of CCL19 (ELC) and one of the two isoforms of CCL21 (SLC) (48). Although it has been well demonstrated that *plt/plt* mice have a gross defect in CCR7 signaling, it should also be mentioned that they retain a pseudogene of CCL19 and one isoform of CCL21, although neither of these has been found to be expressed in the thymus. Collectively, these results demonstrated that CCR7 is dispensable for clonal deletion to ubiquitous self-antigens.

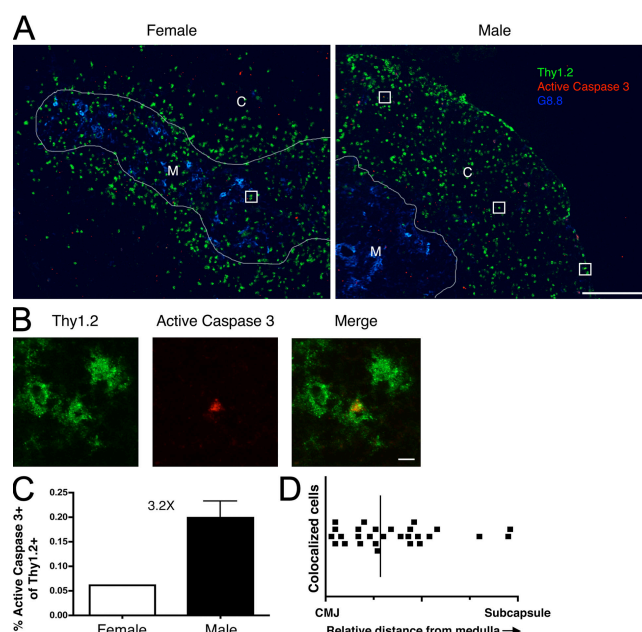
To ultimately determine the anatomical location of thymocytes undergoing clonal deletion, we examined the localization

of apoptotic cells in tissue sections from BM chimeras shown in Fig. 3. In this experiment,  $\text{HY}^{\text{cd}4}$  mice were on the TCR $\alpha^0$  background such that all the Thy1.2 $^+$  cells were male-reactive  $\text{HY}^{\text{cd}4}$  thymocytes. In female mice,  $\text{HY}^{\text{cd}4}$  thymocytes were positively selected and clustered in and around the medulla (Fig. 5 A). However, in male mice  $\text{HY}^{\text{cd}4}$  thymocytes were virtually absent from the medulla, indicating that these cells had either died before they reached the medulla or shortly upon arriving there (Fig. 5 A). To address where  $\text{HY}^{\text{cd}4}$  thymocytes were undergoing clonal deletion, we looked at the colocalization of Thy1.2 $^+$  cells with active Caspase 3 immunoreactivity (Fig. 5 B), which is highly specific for cells undergoing apoptosis (Fig. S4, available at <http://www.jem.org/cgi/content/full/jem.20080866/DC1>). We found that apoptotic  $\text{HY}^{\text{cd}4}$  thymocytes were found throughout the cortex in male mice and not in the medulla (Fig. 5 A, white squares).

To further confirm that the cells we identified were indeed the male-reactive  $\text{HY}^{\text{cd}4}$  thymocytes of interest, we quantified their presence with respect to total Thy1.2 $^+$  cells. Similar to the data obtained by flow cytometry, colocalized cells were



**Figure 4. CCR7 is dispensable for clonal deletion of  $\text{HY}^{\text{cd}4}$  thymocytes.** (A) The expression of CCR7 on female and male T3.70 $^+$  thymocytes from  $\text{HY}^{\text{cd}4}$  mice on a WT or Bim $^o$  background. (B) BM from female  $\text{HY}^{\text{cd}4}$  mice on either WT or CCR7 $^o$  background was mixed with WT female or male BM and transferred into WT female or male recipients. Activation of Caspase 3 in T3.70 $^+$  thymocytes of the indicated genotype is depicted. Data represent the mean from four individuals from one experiment  $\pm$  SD.  $P = 0.0002$  when comparing either WT males and females or CCR7 $^o$  males and females. The difference between WT males and CCR7 $^o$  males was not significant. (C)  $\text{HY}^{\text{cd}4}$  D $^{\text{bo}}$  BM was mixed with WT or *plt/plt* female or male BM and transferred into WT or *plt/plt* female or male mice. The expression of CD69 and PD-1 on T3.70 $^+$  thymocytes from the indicated group is shown. (D) The activation of Caspase 3 on T3.70 $^+$  thymocytes. Data depicts the mean  $\pm$  SD. The fold change is indicated.  $P = 0.0066$ . Data are representative of three separate experiments.



**Figure 5. Colocalization of active Caspase 3 and Thy1.2 identifies male-reactive thymocytes undergoing clonal deletion throughout the cortex.** Thymus tissue sections from mice in Fig. 3 were analyzed by immunofluorescence for Thy1.2, active Caspase 3, and G8.8. (A) Images were overlaid and examined for colocalization between Thy1.2 and active Caspase 3. White lines are drawn around G8.8 $^+$  cells to identify the medulla and boxes identify colocalized cells. C, cortex; M, medulla. Bar, 250  $\mu\text{m}$ . (B) Colocalization was confirmed at high magnification. Bar, 5  $\mu\text{m}$ . (C) The frequency of colocalization is expressed as the number of colocalized cells per total Thy1.2 $^+$  cells in ten different images per thymus. Data represent the mean from four different males  $\pm$  SD. (D) The relative distance of colocalized cells from the medulla was digitally calculated using Photoshop. The drawn line indicates the median of the dataset. The entire figure is representative of two individual groups of chimeras and three replicates of immunofluorescence staining.

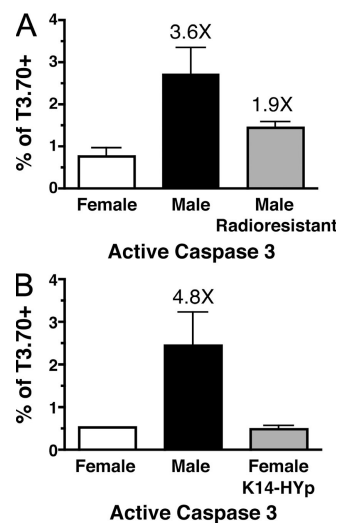
increased in the male mice over female by a similar level (3.2-fold; Fig. 5 C), thereby validating the use of immunofluorescence to identify  $\text{HY}^{\text{cd4}}$  thymocytes undergoing clonal deletion. We quantified the distance of colocalized cells from the medulla, and when normalized measurements from colocalized cells were plotted together (see Materials and methods), thymocytes appeared to be distributed throughout the cortex, even as far out as the subcapsular zone (Fig. 5 D). Importantly, none of the few  $\text{Thy1.2}^+$  cells present in the medulla were active Caspase  $3^+$ . These results confirmed that clonal deletion of  $\text{HY}^{\text{cd4}}$  thymocytes occurs in the cortex. In support of this conclusion, no defect in clonal deletion was observed when we examined mixed BM chimeras using *aly/aly* mutant mice, which have a profound cell-intrinsic defect in medullary thymic epithelial cell (mTEC) development (Fig. S5, available at <http://www.jem.org/cgi/content/full/jem.20080866/DC1>).

#### Apoptosis of $\text{HY}^{\text{cd4}}$ thymocytes is inefficiently induced by radioresistant cells and cTECs but preferentially occurs near DCs

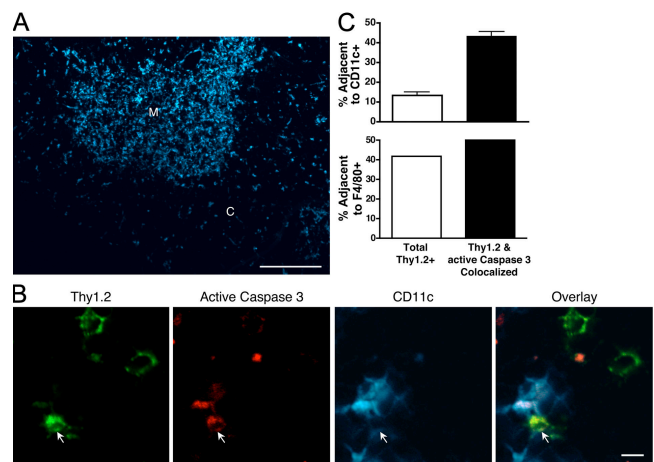
Because the medulla appeared to be dispensable for negative selection of  $\text{HY}^{\text{cd4}}$  thymocytes, we next sought to define fac-

tors present in the cortex that were important for this process. We first examined whether or not clonal deletion is impaired when radioresistant cells are the only competent APC. To do this, we created mixed BM chimeras by adoptively transferring female  $\text{HY}^{\text{cd4}}$   $\text{D}^{\text{bo}}$  BM mixed with nontransgenic female  $\text{D}^{\text{bo}}$  BM into lethally irradiated male B6 mice. This strategy does not allow for potential cross-presentation of male antigen by BM-derived DC (49). When the resulting chimeras were analyzed, a similar up-regulation of CD69 and PD-1 was observed (Fig. S6, A and B, available at <http://www.jem.org/cgi/content/full/jem.20080866/DC1>). However, the activation of Caspase 3 was reduced by half (Fig. 6 A). This result led us to conclude that radioresistant cells are capable of presenting high-affinity antigen to male-reactive cells, but we questioned whether they are efficient at inducing apoptosis.

Because radioresistant cells are a heterogeneous mixture of different cell types, we decided to test the ability of a specific cell type, the cTEC, to induce clonal deletion. To do this, we created mixed BM chimeras by transferring a mixture of female  $\text{HY}^{\text{cd4}}$   $\text{D}^{\text{bo}}$  BM and female B6 BM (or nontransgenic  $\text{D}^{\text{bo}}$  BM [unpublished data]) into irradiated female K14-HYp transgenic mice. K14-HYp mice express the high-affinity male peptide derived from the Y chromosome under the control of the human K14 promoter, which is transcriptionally active in cTECs (37). CD8 SPs did not develop, and a large population of  $\text{T3.70}^+$  cells with a DN phenotype accumulated in these mice (Fig. S6 C). The male-reactive cells had been signaled by their high-affinity antigen, as determined



**Figure 6. Radioresistant cells and cortical epithelial cells inefficiently induce apoptosis.**  $\text{HY}^{\text{cd4}}$   $\text{D}^{\text{bo}}$  female BM was mixed with female or male BM from WT mice and transferred into WT female and male recipients as controls. (A) A cohort of WT male mice reconstituted with a mixture of  $\text{HY}^{\text{cd4}}$   $\text{D}^{\text{bo}}$  female BM and nontransgenic  $\text{D}^{\text{bo}}$  female BM was added. Data are representative of two separate experiments. (B) A cohort of female mice expressing the K14-HYp transgene reconstituted with a mixture of  $\text{HY}^{\text{cd4}}$   $\text{D}^{\text{bo}}$  BM and WT female BM was added. Data are representative of five separate experiments. The frequency of active Caspase  $3^+$   $\text{T3.70}^+$  cells in chimeric mice is shown. Data represent the mean from three to six individuals  $\pm$  SD. Fold change over the female control group is indicated. A,  $P = 0.0463$  when comparing the male radioresistant group to the WT female group and  $P = 0.1191$  when comparing the male radioresistant group to the WT male group; B,  $P = 0.0042$  when comparing WT male and female groups and  $P = 0.0085$  when comparing the WT male group to the female K14-HYp group.



**Figure 7. Male-reactive cells undergoing apoptosis preferentially associate with cortical DCs.** (A) Immunofluorescence staining for CD11c in the thymus. Areas of cortex and medulla are indicated. Bar, 250  $\mu\text{m}$ . (B) Thymus tissue sections from mice in Figs. 3 and 5 were analyzed by immunofluorescence for  $\text{Thy1.2}$ , active Caspase 3, and CD11c. A  $\text{Thy1.2}$  and active Caspase  $3^+$  colocalized cell immediately adjacent to a  $\text{CD11c}^+$  cell is indicated by arrows. Bar, 5  $\mu\text{m}$ . (C) The frequency of cells associated with  $\text{CD11c}^+$  cells was quantified for the indicated subsets. Data represent the mean from four different males  $\pm$  SD.  $P < 0.0001$ . The frequency of cells associated with  $\text{F4/80}^+$  cells is also depicted. Data are representative of two individual groups of chimeras and one set of immunofluorescence staining.

by the up-regulation of CD69 (Fig. S6 D); however, Caspase 3 was not activated at an elevated level compared with the female control (Fig. 6 B). In addition, a high percentage (>90%) of T3.70<sup>+</sup> thymocytes expressed high levels of PD-1, suggesting an increased survival of male-reactive cells (Fig. S6 D). From these results, we conclude that cTECs are capable of delivering a high-affinity signal to male-reactive HY<sup>cd4</sup> thymocytes but are inefficient at inducing apoptosis.

Because cTECs did not appear to be a major APC for inducing clonal deletion, we next examined CD11c<sup>+</sup> DC. Using immunofluorescence microscopy, we saw that the majority of CD11c<sup>+</sup> cells in the thymus were clustered in and around the medulla, as expected. Importantly, we noted the sparse but distinct presence of CD11c<sup>+</sup> cells in the cortex (Fig. 7 A). These cells are distinct from cortical macrophages as judged by the general lack of colocalization with F4/80 (Fig. S7, available at <http://www.jem.org/cgi/content/full/jem.20080866/DC1>). We then stained sections for Thy1.2, active Caspase 3, and CD11c (Fig. 7 B) as described earlier for Fig. 5. We found that 40–50% of apoptotic HY<sup>cd4</sup> cells were immediately adjacent to CD11c<sup>+</sup> cells (Fig. 7, B and C). This association with CD11c<sup>+</sup> cells was a significant increase over the percentage of total (nonapoptotic) HY<sup>cd4</sup> cells in close contact with CD11c<sup>+</sup> cells (Fig. 7 C, top). The colocalized cells immediately adjacent to CD11c<sup>+</sup> cells were found distributed throughout the cortex, as seen in Fig. 5 D. We further examined tissue sections from these mice and found no preferential association of apoptotic HY<sup>cd4</sup> thymocytes with F4/80<sup>+</sup> macrophages (Fig. 7 C, bottom). From these results, we conclude that HY<sup>cd4</sup> thymocytes undergoing clonal deletion preferentially associate with DCs present in the cortex.

To directly test the role of DCs in induction of clonal deletion, we made use of mice that express the diphtheria toxin receptor (DTR) under the control of the CD11c promoter

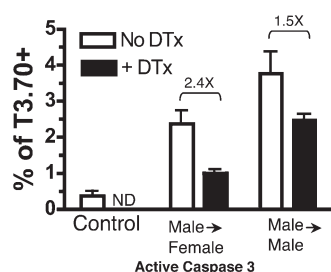
(CD11c-DTR), which allows for the conditional ablation of CD11c<sup>+</sup> DCs upon administration of toxin (50). We generated mixed BM chimeras with HY<sup>cd4</sup> D<sup>bo</sup> and CD11c-DTR BM from male mice. We then transferred this mixed BM into either WT female or male mice. In the first scenario, only BM-derived cells can mediate clonal deletion, whereas in the second case, both BM-derived and radioresistant cells can present the high-affinity ligand. Upon administration of toxin, we achieved >90% depletion of thymic DCs, whereas the frequency of F4/80<sup>+</sup> CD11b<sup>+</sup> macrophages was undiminished (unpublished data). HY-specific thymocytes were activated in both scenarios (Fig. S8, available at <http://www.jem.org/cgi/content/full/jem.20080866/DC1>). Nonetheless, a significant decrease in the activation of Caspase 3 in HY<sup>cd4</sup> cells was observed when DCs were depleted in both chimeras, although the decrease was more substantial in chimeras where only BM-derived cells could induce clonal deletion (Fig. 8). These results provide direct evidence that DCs induce clonal deletion of thymocytes specific to ubiquitous self-antigens in the steady state.

## DISCUSSION

Our data in this paper demonstrate that deletion of T cells with high affinity for ubiquitous self-antigens occurs in the thymic cortex. This is a significant finding because the use of TCR transgenic model systems to establish this point in the past has been challenged because most TCR transgenics express an  $\alpha\beta$  TCR too early in development. However, in this study we used a model where the  $\alpha\beta$  TCR is not expressed until the DN to DP transition. In addition, we generated mixed BM chimeras to reduce the frequency of antigen-specific cells and to avoid disruption of thymic architecture that is typical of TCR transgenics. Therefore, we suggest that polyclonal thymocytes specific for ubiquitous self-antigens would also be deleted in the thymic cortex.

Surprisingly, our results also showed that the induction of apoptosis after TCR activation in DP thymocytes was not very rapid in vivo, allowing for the accumulation of signaled DPs in the steady state. The lag in apoptosis in vivo and subsequent accumulation of signaled DP thymocytes facilitated the analysis of new gene transcription in thymocytes undergoing negative selection in vivo (45). Especially notable was PD-1, which was not up-regulated in female mice and maximally up-regulated in male mice only quite late at 48 h (Fig. 2 B). Although PD-1 is a good marker of high-affinity signaling in this system, it does not seem to be functionally important, as in vivo antibody blockade of PD-1 interaction with its ligands did not impair deletion (unpublished data).

One of the most surprising findings here was the asynchronous nature of deletion in vivo. This is in contrast to the in vitro situation, where the magnitude of apoptosis is much greater and synchronous, with Caspase 3 activation peaking in 8 h of culture (Fig. 2 D). As a potential explanation for the asynchronous nature of clonal deletion in vivo, we examined the possibility that thymocytes require trafficking to the medulla to undergo apoptosis. However, we saw no defect in



**Figure 8. Cortical DCs are efficient APC for inducing clonal deletion.** Female HY<sup>cd4</sup> D<sup>bo</sup> BM was mixed with nontransgenic D<sup>bo</sup> BM or CD11c-DTR female BM and transferred into D<sup>bo</sup> or WT female recipients, respectively, as controls. Female HY<sup>cd4</sup> D<sup>bo</sup> BM was also mixed with male CD11c-DTR BM and transferred into WT females or males and either left untreated or treated with DTx. Activation of Caspase 3 in treated versus untreated T3.70<sup>+</sup> thymocytes of the indicated group is depicted. Data represent the mean from three to five mice  $\pm$  SD. The fold change comparing untreated and DTx-treated mice for each group is indicated.  $P < 0.0001$  for the male into female group and  $P = 0.0242$  for the male into male group. ND, not done. Data are representative of four separate experiments.



clonal deletion in the absence of CCR7 or its ligands (Fig. 4) or when mature and organized mTECs were absent in *aly/aly* mice (Fig. S5). In addition, male-reactive thymocytes initiated apoptosis at sites throughout the cortex and were absent from the medulla altogether (Fig. 5).

An alternative hypothesis to account for the asynchronicity of deletion *in vivo* is that TCR-signaled cells must interact with a second cell for efficient induction of apoptosis. In fact, we show that male-reactive thymocytes undergoing apoptosis preferentially associate with cortical DCs (Fig. 7) and that conditional ablation of DCs caused a marked reduction in the efficiency of clonal deletion (Fig. 8). Recently, Ellen Robey's group has also defined and studied this cortical DC population (Robey, E., personal communication). They used two-photon imaging to demonstrate that after positive selection, DP thymocytes increase their motility and preferentially associate with cortical DC. Our results in this paper would suggest that this increased motility favors the induction of tolerance to ubiquitous self-antigens.

The final novel finding of this study is that cTECs are not efficient APC for inducing apoptosis. At first glance, this may appear to contradict data that showed immunological tolerance to antigens expressed exclusively by cTECs (40). However, we note that despite the fact that apoptosis of  $\text{HY}^{\text{cd4}}$  thymocytes was not induced in K14-HYP mice, neither was differentiation into mature CD8 SP. The lack of mature CD8 SP ultimately represents immunological tolerance, even if the mechanism is not clonal deletion. This observation could be seen to support a model where low-affinity ligands provide a unique signal for positive selection that high-affinity ligands cannot recapitulate even if apoptosis is blocked, a notion for which there is other evidence in the field (51).

In summary, we used a modified  $\text{HY}^{\text{cd4}}$  TCR transgenic model to study when and where clonal deletion to ubiquitous self-antigens occurs. We found that even when TCR expression is delayed until the DP stage, clonal deletion occurs in the cortex. Cortical epithelial cells, although probably representing the major stromal cell type that thymocytes interact with in the cortex, are not a major player in the induction of apoptosis. Instead we defined a novel population of DCs in the cortex of the thymus and showed that these cells induce antigen specific clonal deletion.

## MATERIALS AND METHODS

**Mice.**  $\text{HY}^{\text{cd4}}$  and  $\text{HY}^{\text{cd4}}$   $\text{TCR}\alpha^0$  have been described previously (41), as were K14-HYP mice (37).  $\text{HY}^{\text{cd4}}$   $\text{D}^{\text{bo}}$  were generated by intercrossing  $\text{HY}^{\text{cd4}}$  mice with H-2  $\text{D}^{\text{bo}}$  mice (Taconic).  $\text{HY}^{\text{cd4}}$   $\text{Bim}^0$  mice were generated by intercrossing  $\text{HY}^{\text{cd4}}$  mice with  $\text{Bim}^0$  mice (provided by B. Blazar, University of Minnesota, Minneapolis, MN).  $\text{HY}^{\text{cd4}}$   $\text{CCR7}^0$  mice were generated by intercrossing  $\text{HY}^{\text{cd4}}$  mice with  $\text{CCR7}^0$  mice (Jackson ImmunoResearch Laboratory). *Plt/plt* mice were provided by T. Randall (Trudeau Institute, Saranac Lake, NY). *Aly/aly* mice were a gift from M. Schlomchik (Yale University, New Haven, CT). C57BL/6 (B6) mice were purchased from National Cancer Institute, and C57BL/6-Thy1.1 (B6.PL) mice were purchased from the Jackson ImmunoResearch Laboratory, as were CD11c-DTR mice. All animals were maintained and treated in accordance with federal guidelines approved by the University of Minnesota Institutional Animal Care Committee.

**Flow cytometry.** All fluorochrome-conjugated and biotinylated antibodies were purchased from BD Biosciences, eBioscience, Invitrogen, or BioLegend, except for anti-active Caspase 3 (Asp175), which was purchased from Cell Signaling Technology, and anti-BrdU (PRB-1), which was purchased from Phoenix Flow Systems. Detection of CCR7 was accomplished using an ELC-Fc fusion protein (provided by J. Cyster, University of California, San Francisco, San Francisco, CA), followed by biotin-labeled anti-human Fc antibody (Jackson ImmunoResearch Laboratories) and streptavidin-labeled APC. Conventional surface staining was performed by staining cells with antibody for 30 min on ice in FACS buffer (PBS, 1% FCS, and 0.02% azide, pH 7.2) and washing two times in FACS buffer after each antibody incubation. Intracellular staining for active Caspase 3 was performed by fixation and permeabilization using 0.5% formaldehyde for 10 min at 37°C and 90% methanol for 30 min on ice. Cells were then incubated with anti-active Caspase 3 primary antibody at a concentration of 1:100 in 0.5% BSA in PBS for 30 min at room temperature, washed twice, and incubated with goat anti-rabbit IgG Alexa Fluor 647 for 30 min at room temperature. Cell events were collected using an LSR-II cytometer (BD Biosciences) and analyzed with FlowJo software (Tree Star, Inc.).

**BM chimeras.** Recipient mice were lethally irradiated with 1,000 rads and, for experiments using  $\text{HY}^{\text{cd4}}$   $\text{D}^{\text{bo}}$  donors, were also depleted of NK cells by i.p. injection of 50  $\mu\text{g}$  PK136 days  $-2$  and  $-1$  before reconstitution and again with 25  $\mu\text{g}$  1 and 2 wk after. Recipient mice were then reconstituted with  $5\text{--}10 \times 10^6$  total BM cells depleted of T and NK cells and kept on antibiotic water for 3 wk. All chimeras were analyzed after 8 wk unless otherwise indicated.

**BrdU labeling.** BrdU time courses were performed by injecting 100  $\mu\text{l}$  of 10 mg/ml BrdU (B5002; Sigma-Aldrich) twice at half-hour intervals for a total of 2 mg/mouse at the indicated time point before harvest. Thymi were harvested and surface stained as described, followed by intracellular staining for BrdU and active Caspase 3 using the APC BrdU flow kit (BD Biosciences) with the substitution of the anti-BrdU antibody PRB-1.

**Immunofluorescence.** Thymi were embedded in O.C.T. Compound (Tissue-Tek), snap frozen in a dry ice bath, and stored at  $-80^\circ\text{C}$ . 10- $\mu\text{M}$  frozen sections were cut using a CM1800 cryostat (Leica), fixed with acetone for 10 min at room temperature, and then stained using the TSA Fluorescence Systems kit (PerkinElmer). Biotinylated Thy1.2 was visualized with streptavidin (SA)-Alexa Fluor 488. G8.8 (provided by A. Farr, University of Washington, Seattle, WA) was visualized with goat anti-rat IgG Alexa Fluor 647 (Invitrogen). Antiactive Caspase 3 (Asp175; Cell Signaling Technology) was visualized, after overnight incubation at  $4^\circ\text{C}$  at 1:50 concentration, with goat anti-rabbit IgG Alexa Fluor 555 (Invitrogen). Biotinylated anti-CD11c and anti-F4/80 (BM8) were visualized using SA-conjugated tyramide followed by horseradish peroxidase-coupled Cy5. anti-K5 (Covance) was visualized with goat anti-rabbit Alexa Fluor 555. MTS-10 (provided by R. Boyd, Monash University, Melbourne, Victoria, Australia) was visualized with goat anti-rat IgM Alexa Fluor 647. Biotinylated UEA-1 (Vector Laboratories) was visualized with SA-Alexa Fluor 488. Images were obtained using a microscope (AX70; Olympus) with a camera (MRC 1024; Bio-Rad Laboratories) operating with the LaserSharp software (Bio-Rad Laboratories) and analyzed using ImageJ (National Institutes of Health) and Photoshop (Adobe) software.

**Quantification and measurement of colocalization.** Images were adjusted to maximize brightness, while avoiding saturation, and to minimize background based on negative staining controls. The total number of colocalized cells in 10 images per mouse was divided by the total number of Thy1.2 $^{+}$  cells in all 10 images. The total number of Thy1.2 $^{+}$  cells was determined by electronically counting the total number of pixels representing Thy1.2 immunoreactivity and dividing by the mean number of pixels/cell. The distance of colocalized cells from the medulla was electronically measured, along with the shortest total distance from the medulla to the capsule



in a straight line intersecting the colocalized cell. The relative distance from the medulla is expressed as ratio of these two measurements.

**Stimulation of thymocytes in vitro.** Thymocytes from female HY<sup>cd4</sup> mice were harvested and cultured in triplicate in RPMI containing antibiotics and 10% serum for 8 h with or without 1  $\mu$ M HYp peptide. Cells were then washed and stained for flow cytometry as described in Flow cytometry. Antigen-specific death was normalized to the frequency of active Caspase 3<sup>+</sup> cells in the unstimulated control.

**Administration of diphtheria toxin (DTx).** Mice were injected i.v. with 100 ng DTx on days -1, -3, -5, -7, and -9 before harvest.

**Statistical methods.** Standard deviation and p-values were determined using Prism software (GraphPad Software, Inc.). P-values were calculated using a two-tailed unpaired *t* test with 95% confidence interval.

**Online supplemental material.** Fig. S1 shows the phenotype of HY<sup>cd4</sup> Bim<sup>o</sup> mice. Fig. S2 shows the phenotype of HY<sup>cd4</sup> D<sup>bo</sup> mice. Fig. S3 shows additional phenotype data of HY<sup>cd4</sup> CCR7<sup>o</sup> mice. Fig. S4 shows immunofluorescence staining controls for active Caspase 3. Fig. S5 shows the medullary dysplasia in *aly/aly* mice and that clonal deletion is normal in these mice. Fig. S6 shows additional phenotype data to support Fig. 6. Fig. S7 shows immunofluorescence staining of CD11c and F4/80, with medulla outlined in white. Fig. S8 shows additional phenotype data from CD11c-DTR chimeras. Online supplemental material is available at <http://www.jem.org/cgi/content/full/jem.20080866/DC1>.

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